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Abstract

Clinically effective gene therapy for Cystic Fibrosis has been a goal for over 20 years. A plasmid vector (pGM169) that generates persistent expression and reduced host inflammatory responses in mice has raised prospects for translation to the clinic. The UK CF Gene Therapy Consortium is currently evaluating long-term repeated delivery of pGM169 complexed with the cationic lipid GL67A in a large Multidose Trial. This regulatory-compliant evaluation of aerosol administration of nine doses of pGM169/GL67A at monthly intervals, to the sheep lung, was performed in preparation for the Multidose Trial. All sheep tolerated treatment well with no adverse effects on haematology, serum chemistry, lung function or histopathology. Acute responses were observed in relation to bronchoalveolar cellularity comprising increased neutrophils and macrophage numbers 1 day post-delivery but these increases were transient and returned to baseline. Importantly there was no cumulative inflammatory effect or lung remodelling with successive doses. Molecular analysis confirmed delivery of pGM169 DNA to the airways and pGM169-specific mRNA was detected in bronchial brushing samples at day 1 following doses 1, 5 and 9. In conclusion, nine doses of pGM169/GL67A were well tolerated with no significant evidence of toxicity that would preclude adoption of a similar strategy in CF patients.

Keywords: Gene Transfer, Lipid, Lung, Epithelium

1. Introduction

The cloning of the Cystic Fibrosis Transmembrance Conductance Regulator (*CFTR)* gene in 1989 sparked significant interest in the development of gene therapy for cystic fibrosis (CF) to the extent that CF was regarded as the gene therapy paradigm. Twenty four years and approximately 25 gene therapy clinical trials later [1], CF remains a disease where, for the majority of patients, the only treatments available are those that delay the inevitable and ultimately fatal decline in lung function.

The UK CF Gene Therapy Consortium (GTC) has continued to search for solutions to overcome some of the problems associated with gene therapy, developing non-viral formulations with improved plasmid DNA constructs that reduce inflammatory responses and increase the duration of expression in preclinical models [2]. A plasmid (pGM169) has been engineered that is free of CG dinucleotides and contains a codon- optimised *CFTR* cDNA driven by the hCEFI promoter (Elongation Factor 1a promoter with a Human Cytomegalovirus (CMV) enhancer and a synthetic Intron). Our preclinical programme identified the cationic liposome GL67A as the most efficient non-viral vector for delivery of the plasmid DNA to the lung [3]. Data from a single dose Phase IIa trial of pGM169/GL67A complexes in CF patients indicated that delivery was safe and in some patients demonstrated evidence of changes in transepithelial chloride conductance towards, or into, the normal range which could persist for up to 13 weeks [4]. A question that has yet to be answered is whether the relatively low levels of expression of the delivered gene, produced by non-viral gene therapy [5], can lead to clinical benefit for CF patients ie. Improve lung function, if expression can be sustained long term. This question is particularly relevant considering the endogenously very low levels of normal CFTR expression in the lungs, and the comparatively large clinical benefit CF patients with even small degrees of residual CFTR expression demonstrate. This fact has led us, (the GTC), to conclude that until a Multidose Trial of sufficient duration is undertaken, this key question cannot be addressed. Based on data showing the duration of transgene expression in mice [2] and our single-dose trial [4] the Multidose trial includes dosing at 4-week intervals over the course of a year.

Since it has previously been shown that a single dose of pDNA/GL67A can result in a transient flu-like syndrome in patients [5, 6] and a transient increase in bronchoalveolar lavage neutrophils in our

sheep model [3], it is of key importance to demonstrate that none of these mild and transient responses are exacerbated by repeated administration. Further, we wished to ensure there was no accumulation of lipid-filled macrophages, or significant remodelling of lung architecture. In preparation for the Multidose clinical trial, we have therefore conducted this toxicology study (TS) in sheep - an animal with lung structure and size similar to that of humans.

The main objective of this sheep TS was to assess the toxicity of repeated doses of aerosolised pGM169/GL67A complexes. A 10ml dose was selected as this volume is the maximal dose being considered for application in the Multidose Trial. Nine doses were delivered over a 32-week period at intervals of 4 weeks in line with the EMA guidelines (European Medicines Agency CPMP/ICH/286/95)for minimum duration in support of a clinical study of longer than 6 months. Using translation-relevant toxicity indices we assessed whether there was any evidence of cumulative local or systemic toxicity, We also took this opportunity to evaluate pGM169 plasmid delivery and expression in the lungs using this system.

2. Materials and methods

2.1 Animals

All experiments were carried out with approval of appropriate local Ethics Committees and according to Home Office regulations. Suffolk Cross lambs, average 33-53 kg (average 42kg), were treated on arrival with a parasiticide (Cydectin) and a flukicide (Fasinex), both administered at the manufacturers recommended dosage. A repeat dose of Cydectin was administered approximately 14 days later and then again 6 months later, again following the manufacturers recommended dose rate for each individual animal (0.1 ml /5 kg bodyweight). They were also treated with a Clostridial and Pasturella vaccine (Heptavac P) at the manufacturer's recommended dose during the trial to comply with standard Roslin Institute animal husbandry. The animals underwent a preliminary examination involving bronchoscopic visualisation and bronchoalveolar lavage (BAL) under gaseous anaesthesia prior to treatment to confirm the absence of pre-existing pulmonary disease. This preliminary BAL procedure was restricted to the accessory lobe (Right Apical, RA) which branches from the trachea proximal to the carina. The time between preliminary BAL and delivery was 2 weeks to minimize the likelihood of any effect on gene transfer. Four castrated male and four females were randomly assigned to the active multidose gene therapy (MD_GT) group and the anaesthetic alone control (MD_CON) group respectively. The animals were housed in four groups of four with two additional 'sentinel' (SEN) animals assigned to each pen to monitor health status since the accommodation was not pathogen-free. Pre-treatment bronchial brushings (BBr, n=4 per sheep), baseline blood sample and spirometry data were collected from all 24 animals. MD_GT and MD_CON animals were sampled as described in Figure 1 (additional detail in Supplementary Material online, Tables S1-S2). Blood and BAL samples were also collected from SEN animals at times corresponding to Doses 1, 5 and 9. A full set of measurements and samples was collected from SEN animals at necropsy.

2.2 Gene Transfer Agent

This study employed GLP grade plasmid pGM169 (VGXi, Houston, Texas, USA), Lot#pGM169.07C006 (5.1mg/ml) and Lot#pGM169.07F017(5.3mg/ml) and GL67A (Octoplus, Leiden,

The Netherlands) Batch# 08K26701-01A. pGM169 contains a codon- optimised,CpG-free CFTR cDNA driven by the hCEFI promoter which is composed of the CpG-free Human CMV enhancer, the CpG-free Elongation Factor 1a promoter together with a CpG-free synthetic Intron.

2.3 Aerosol delivery

Anaesthetised animals were dosed, by whole lung aerosol exposure via an endotracheal tube, once every 4 weeks for 32 weeks giving a duration of 9 months. This is consistent with the EMA guidelines for minimum duration in support of a clinical study of longer than 6 months. Anaesthetic controls were intubated and exposed to room air with ambient humidity for 1h to avoid any drying effect of bottled gas. This study employed a well characterised lung aerosol delivery model in which gene transfer agents aerosolised using PARI LC Plus nebulisers are delivered to anaesthetised sheep via an endotracheal tube using a negative pressure ventilation system [3, 7]. The pGM169/GL67A formulations were prepared as described [8] by mixing 5ml of pGM169 with 5 ml of GL67A and consisted of 2.64mg/mL plasmid DNA, and approximately 14.29mg/ml total lipid (GL67, DOPE, DMPE-PEG 5000) for a total of 16.93mg/mL combined test item product. This is equivalent to 8mM plasmid DNA and 6mM GL67A [9]. Animals placed in sternal recumbency in a whole body respirator were exposed to 10ml of aerosolised pGM169/GL67A delivered via an endotracheal tube.

2.4 Lung function analysis

Airway pressures were measured by connecting one side of a differential pressure transducer to a side port at the proximal end of the endotracheal tube, the other side of which was open to the atmosphere. Respiratory air flows were measured by a screen pneumotachograph linear to 5.0 L·s-1 (F300L Mercury Electronics, UK) connected to the proximal end of the endotracheal tube. This pneumotachograph was connected to a sensitive differential pressure transducer. Flow and pressure transducers were calibrated by rotameter (KDG 2000, KDG Mobrey, Crawley, UK) and U-tube manometers respectively. Lung resistance and dynamic lung compliance, lung diffusion capacity [DLCO or transfer Factor TLCO], alveolar lung volume (VAeff), functional residual capacity (FRC) were all determined immediately pre-and post-exposure. DLCO measurements determine the

efficiency of the lung at diffusion across the alveolar-capillary membrane. This test involved inhalation of air containing carbon monoxide (0.28%) and helium (14%) and breath held for 10 seconds, then rapidly exhaled. The exhaled gas from the single breath was analyzed on a Morgan Benchmark gas analyser to determine carbon monoxide absorption during the breath to assess diffusion capacity and the final exhaled concentration of helium to determine VAeff and FRC. DLCO (SB) data are adjusted for haemoglobin levels. Forced Expiration data were obtained using a 210 L steel drum serving as a vacuum reservoir necessary to elicit a forced expiration in the anaesthetized sheep. Connection between the reservoir and the proximal end of the endotracheal tube was effected by thick walled wide bore tubing with the timing and duration of connection controlled by manual operation of a large bore (19mm) fast acting (40ms) solenoid valve (Danfoss EV210B, M & M Controls, Manchester, UK). Vacuum pressure within the drum was achieved through operation of a vacuum pump and could be adjusted to and maintained at the appropriate level (-4 kPa; visualized using a U-tube manometer) by manually operated bleed and check valves. Following a period of tidal ventilation the Cuirass pump was disconnected from the negative pressure ventilator and the sheep allowed to exhale passively to functional residual capacity (FRC). Thereafter the lungs were slowly inflated to an airway pressure of 4kPa and then allowed to passively exhale to FRC. The lungs were then again inflated to a pressure of 4kPa and the proximal end of the endotracheal tube connected to the vacuum reservoir. Solenoid actuation effected the rapid discharge of the lung vital capacity into the vacuum reservoir after which the solenoid was closed. The lungs, by which time were at residual volume, were then gradually re-inflated to an airway pressure of 3kPa, before the forced vital capacity manoeuvre was repeated on a further two occasions. Signals derived from the pneumotachograph and inflation pressure were fed into a computer sampling at 512 Hz through an analog to digital conversion card (NI PCI-6221, National Instruments, TX, USA). A custom designed computer program (LabView, National Instruments, TX, USA) facilitated the real-time visualization of signals, data storage and the subsequent interactive analysis of flow-volume curves. Volume was obtained by digital integration of the flow signal from the pneumotachograph. Post-hoc flow signal processing consisted of 4th-order Butterworth low-pass (15Hz) filtering. Forced vital capacity (FVC) was calculated as the expired volume between the lung volume at +4kPa inspiratory pressure and residual volume. As well as the maximal expiratory flow recorded at the start of expiration (MEF), the maximal expiratory flows at 75%, 50%, 25% and 10% FVC near-residual volume (MEF75, MEF50, MEF25 and MEF10

respectively) were measured, as were the forced expired volumes in 0.5 and 1 sec (FEV0.5 and FEV1) and the ratios formed by their relationship with FVC (FEV0.5/FVC and FEV1/FVC). The average flow between 75%, and 25% FVC near-residual volume was also measured (FEF25-75) as was a rate constant between 50% and 25% FVC near-residual volume (RC50-25; calculated as (MEF50 – MEF25)/(MEV50- MEV25) where MEV50 and MEV25 are the lung volumes at 50% and 25% FVC near-residual volume). Forced ventilation parameters including Maximum and Average Expired Flow (MEF, AEF), Total Expired Volume (FVC) and Forced Expiratory Volume (FEV) were measured before each of the nine doses, at days 1 and 15 post-Dose 1, 5 and 9 and at 4 weeks after the final dose.

2.5 Multiple Breath Washout/Lung Clearance Index

Inert gas multiple breath washout (MBW) involves continuously breathing in a small concentration of tracer gas over a short period of time until equilibrium is reached and then breathing in air while monitoring the concentration of the tracer gas during each expiration until the tracer gas is virtually undetectable in the exhalate [10]. Measuring gas flow concurrently with gas concentration allows various indices to be derived from the MBW data, including the Functional Residual Capacity (FRC) and the Lung Clearance Index (LCI). LCI is a measure of the unevenness of the distribution of ventilation, also called ventilation inhomogeneity. The higher the LCI, the greater the ventilation inhomogeneity. The protocol was adapted from the standard operating procedure currently used in a Single Dose Clinical Trial in humans. The tracer gas used in the MD Toxicology study was sulphur hexafluoride (SF6) at a concentration of 0.2% (supplied by BOC Industrial Gases, UK). An Innocor device was used to measure flow and the gas concentrations of SF6, CO_2 & O_2, with a frequency of 100Hz, using its differential pressure transducer, photoacoustic gas analyser and oxigraph. The Innocor's differential pressure transducer was connected via flexible gas tubes to a Series 4700A Hans Rudolph non-heated linear pneumotachometer. The pneumotachometer was modified to incorporate a gas sampling line, connected to the Innocor via Nafion tubing. A single-use antimicrobial filter was placed between the sheep's endotracheal (ET) tube and the pneumotachometer for each test. The Innocor's flowmeter was calibrated and the flow-gas delay calculated prior to each test session.

MBW was performed following a period of stabilisation of negative-pressure ventilator parameters after the above Lung Function Tests had been performed and an anti-microbial filter had been fitted to the ET tube. The main targets for ventilation were tidal volume equal to 10ml/ kg and end tidal CO_2 ($ETCO_2$ between 4.5% and 5%. The MBW test session was started by attaching the modified pneumotachometer/gas sampling line to the filter and disconnecting the ET tube $ETCO_2$ sampling line. $ETCO_2$ was from then until the end of the MBW test session monitored with the Innocor machine. The pneumotach/gas line assembly was then attached to a flow-past circuit of air containing 0.2% SF6. End expiratory SF6 concentration was monitored in real time on the Innocor screen. The flow-past was disconnected during expiration once the inspiratory and expiratory SF6 concentration difference was less than 0.002%. The Innocor then continued to record data until the absolute expiratory SF concentration was less than 0.002% for more than two consecutive breaths. This represented the end of one MBW test. Two further tests were performed immediately after the first. The raw data were retrieved from the Innocor hard drive and analysed in a standardised way using customised GTC software to integrate the flow and SF6 concentration curves for each breath of each MBW to derive FRC and LCI. Assessment was performed immediately prior to the delivery of Dose 9 and then again immediately prior to PME after the 28day recovery period post-Dose 9. The sentinel group was also assessed immediately prior to PME.

2.6 Sample collection from anaesthetised animals

Bronchoalveolar lavage (BAL) fluid and bronchial brushing (BBr) samples were collected as previously described [3] at the timepoints shown in Fig. 1 and listed in Supplementary Table S2.

2.7 RNA and DNA extraction

Bronchial brushings (BBr) harvested from the airways at at 1 day after Dose 1, 5 and 9 and parenchyma (Tx) and BBr samples taken at post-mortem examination at day 28 after Dose 9 (see Necropsy section) were processed for mRNA and DNA analysis. Total DNA and RNA were prepared from BBr and Lysing Matrix D-homogenised Tx samples using the AllPrep DNA/RNA Mini kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. DNA was eluted from the AllPrep column

in 100µl nuclease-free water and final DNA concentrations were quantified with the Quant-iTPicoGreen dsDNA assay kit (Invitrogen, Paisley, UK). After on-column DNase digestion (Qiagen), RNA was eluted from RNeasy columns in 30 – 50µl nuclease free water (Promega, Southampton, UK) and then further treated with the DNA-free kit (Ambion) according to manufacturer's instructions in order to ensure complete removal of plasmid DNA. Final RNA concentrations were quantified with the Quant-IT RiboGreen RNA assay kit (Invitrogen).

2.8 DNA qPCR analysis

The sequences of the pGM169 DNA TaqMan assay primers were – forward primer (50nM): 169_DNA-5095F (5'-GGAACAGCTCCAAGTGCAAGA-3'), reverse primer (900nM): 169_DNA-5174R (5'-CCTGGTGTCCTGCACTTCCT-3'), and fluorogenic probe (100nM): 169_DNA-5117A (5'-FAM-CAAGCCCCAGATTGCTGCCCTG-TAMRA-3'). The oCFTR assay is specific for DNA detection as it spans a sequence from intron 3 of oCFTR. The sequences of the oCFTR DNA TaqMan assay primers were – forward primer: oCFTRint3-696F (300nM) (5'- TCTTCATGCCTCTGGGAGTGA-3'), reverse primer (300nM): oCFTRint3-778R (5'- AGCACAAGTTCTTAAAGCAATGCA-3') and fluorogenic probe (100nM): oCFTRint3-731T (5'-FAM-CTCCCCAAGAGGTGCCCAGCAAG-NFQ-MGB-3') (NFQ-MGB is dihydrocyclopyrroloindole tripeptide, a non-fluorescent quencher that binds in the minor groove of DNA). Multiplex reactions (both primer sets in each well) were performed in quadruplate, each containing 2µl of DNA diluted to 0.5ng/µl (necropsy samples) or 0.05ng/µl (live bronchial brushings). PCR and fluorescence detection was performed in a final volume of 10µl 1X TaqMan Universal PCR buffer using standard PCR cycling conditions on a TaqMan 7900HT Real Time PCR System (Applied Biosystems). Results were analysed using Sequence Detection System Software Version 2.3. The experimentally determined copy numbers of pGM169-specific DNA (based on a standard curve made from a pGM169 plasmid dilution series) and endogenous oCFTR DNA (on a standard curve made from a dilution series of ovine genomic DNA) were used to calculate DNA in samples, expressed as %vector/endogenous (copies of pGM169 DNA/copies of ovine *CFTR* DNA x 100).

2.9 RNA qPCR analysis

Samples were analysed by TaqMan RT-PCR with primers for amplification of both pGM169-derived mRNA and endogenous oCFTR mRNA as described previously [11], using target-specific primer sets [12] except that cDNA synthesis was performed in 10µl (oCFTR) or 20µl (pGM169) volumes, incubated at 48°C for 30 minutes and 95°C for 5 minutes in a GeneAmp PCR 2700 System (Applied Biosystems), with TaqMan reactions in a final volume of 10µl. Comparator samples included those in which lung RNA was replaced with water (no template control), and synthetic RNA standards ranging from 5 copies/µl to 78,125 copies/µl input RNA. The pGM169 assay is specific for RNA detection as the reverse primer is designed across the intron within the 5' non-coding sequences of pGM169. The ovine CFTR assay is specific for RNA detection as it spans the junction between exons 1 and 2 of ovine CFTR. PCR and fluorescence detection was performed in a final volume of 10µl 1X TaqMan Universal PCR buffer using standard PCR cycling conditions on a TaqMan 7900HT Real Time PCR System (Applied Biosystems). Results were analysed using Sequence Detection System Software Version 2.2.2 or 2.3 (Applied Biosystems).

2.10 Necropsy

Following euthanasia by lethal injection and exsanguination, the lungs were removed for tissue collection as previously described [3] with a few modifications. The following lung segment nomenclature was used: RA, right apical; RI, right intermediate; RC, right cardiac; RVD1, RVD2, right ventral diaphragmatic 1and 2; RCD, right caudal diaphragmatic; LC, left cardiac (cardiac segment of the apicocardiac lobe); LVD1, LVD2, left ventral diaphragmatic 1 and 2; LCD, left caudal diaphragmatic. The pulmonary circulation was flushed out via the pulmonary artery with 2–3 L of PBS before sampling. Organ appearance and weights were recorded. BAL samples were collected from RCD & LC anterior portion. BBr samples were collected from segments RA-anterior, RA-posterior, RC, RI, LC, LVD1, LVD2, LCD. The right side main bronchus was cannulated distal to RI/RC segments for inflation fixing of remaining intact right lung (Segments RVD1, RVD2 and RCD) with formalin for histology and Massons Trichrome stain. Segments RC and LC were inflated with OCT/sucrose and frozen for IHC and Oil Red O stain. Segments RA, LVD1, LCD (AWU, PU, M, L) were finely minced and stored in RNALater for DNA and RNA extraction. After 48hr in formalin the

right lung portion was sliced transversely (~1cm thick) numbered from distal to proximal and four blocks selected for histology. These were two blocks from slice 2 (one with airways and one parenchyma/pleura) and two blocks from the “middle” slice (one with airways and one parenchyma/pleura). Remaining slices were stored in formalin in case more comprehensive analysis was required. One section from each of four different blocks from the inflation fixed right side of the lung was examined histologically in a blinded manner. Samples from other organs were collected as described in Table S2. Detailed procedures for sampling heart, liver, kidney, spleen are listed in Supplementary Materials & Methods.

2.11 Lung Morphometry

Masson’s Trichrome stained slides with samples of lung were imaged using an Olympus BX41 microscope and Olympus Cell ^D Software. Five images of parenchyma from each of two slides per sheep at 40x magnification with a set scale of 50um etched onto them were blinded for analysis. The Image J programme was used to measure the scale bar and set the known length in pixels for that line. Thereafter the length of any line drawn on the image can be calculated. Images were converted to binary to allow %black/white to be calculated. Mean linear intercept (Lm), Area Fraction, Septal Wall Thickness and number of intersections were calculated. The detailed procedure is described in Supplementary Materials & Methods.

2.12 Statistical analysis

The study essentially consists of self- contained repeated units of 28 days- in terms of the majority of the datasets. For every 28 day unit there are; variables measured from observations not requiring anaesthesia and variables measured from observations requiring anaesthesia. For the majority of the analyses use is made of the statistical technique ‘linear mixed-effect (LME) models‘. This system of analyses takes into account the repeated measurements/units from each sheep (with the number of units increasing during the study). The use of LME allows for any individual sheep effects to be taken into account as a random factor (without, formally, being “penalised” in terms of degrees of freedom). Differences between MD_CON, MD_GT and Sentinel animals and differences with increasing doses

are then evaluated as fixed effects. For analysis of one blood parameter (Total Monocytes), normalisation of the residuals was not possible therefore simple non-parametric comparisons were carried out as a - Wilcoxon signed rank test for paired (eg Pre v Post), and Mann-Whitney test for two groups (e.g. MD_CON versus MD_GT). LME models were also used to evaluate % vector/endogeneous from bronchial brushings (BBr) and tissue (Tx) samples. For this analysis the few (n=9) positive but not quantifiable (PBNQ) and not determined (ND) observed at D1d1 and D9d1 were excluded. For the data from the MBW procedure (LCI & FRC) a one-way ANOVA was carried out with standard post hoc Tukey pairwise comparisons considered to look to see where differences between groups (MD_CON, MD_GT and sentinels) and time points occurred. The same technique was used to look at differences between groups in septal wall thickness and the ratio between lung tissue and airspace (Area %). For mean linear intercept (Lm) the residuals from the ANOVA were not normal so the non-parametric equivalent (Kruskal-Wallis and the *post hoc* Dwass-Steel-Chritchlow-Fligner tests) was carried out. In all cases *** $p<0.001$, ** $p<0.01$, * $p<0.05$, , and all analyses were carried out in R (v3.0.0 © 2013 The R Foundation for Statistical Computing). The Null Hypothesis was rejected at $p<0.05$..

Graphing conventions. For the BAL cell data (Fig. 3), lung morphometry data (Fig. 7) and MBW data (Fig. S12) each point is the value for a single animal and the bar represents the median value. For the plasmid DNA copy number data (Fig. 8) each point represents an individual sample and the bar represents the median value. For all the other figures each point represents the mean ± 95% Confidence Interval for the anaesthesia only control group, the GL67A/pGM169 treated group and the Sentinel group.

3. Results

3.1 Dose and delivery

Three groups of animals (n=8 each) were included in the study. One group received multiple doses of the gene therapy formulation (MD_GT); a second group was exposed to multiple periods of room air only to control for the repeated anaesthetic periods (MD_CON). Figure 1 illustrates the study

design for these two groups. Each monthly 10ml dose contained 26.4mg pGM169 and approximately 150mg lipid. The average sheep bodyweight over the 9 doses was 54.7kg and thus the average dose was 0.5 mg/kg pGM169 and 2.6 mg GL67A / kg . Average delivery time (+/- Standard Deviation (SD)) for the MD_GT Group was 67min +/- 9min and the average amount aerosolised (obtained by subtracting final weight of nebuliser from starting weight) was 86.2 +/- 1.5% of the 10ml dose. The control group (MD_CON) was exposed to room air for 60 min.

3.2 Systemic toxicity parameters

Physiological and behavioural signs before and after dosing indicated that the repeated delivery was well tolerated with no discernible effects on appetite, condition or demeanour. There were no consistent differences in body temperature between control (MD_CON) and treated (MD_GT) groups (*P*= 0.107 Fig. S1). In the MD_GT group, the only significant changes in temperature from pre-treatment to day 1 were after Doses 4 (D4d1) and 6 (D6d1) (median change 0.25°C, $p<0.031$) but the median changes were small and transient (D4d1 +0.5°C, D6d1 +0.25°C). Animals in both groups gained weight at a rate equivalent to each other and to the sentinel animals over the course of the study and no differences between MD_GT, MD_CON and sentinels were present at post-mortem (*P*>0.125, Fig. S2).

Red blood cell (RBC) numbers, haemoglobin and peripheral white blood cell (WBC) counts were elevated at day 1 post-treatment for each dose in both MD_CON and MD_GT groups (data not shown). The increase in WBCs was higher for the MD_GT group (*P*=0.036), however changes in all these parameters were mild and resolved between doses. As expected, the leucocytosis was reflected in significant changes in blood neutrophil numbers (Fig. S3) from pre-treatment to day 1 at 5/9 doses in MD_GT and 3/9 doses in the MD_CON groups but these changes were small and had resolved by the day 15 measurment (*P*>0.153). These acute changes did not increase in severity with successive doses in either group of animals (*P*>0.343). No consistent or cumulative effect was observed on blood monocytes or eosinophils.

Significant increases in the serum acute phase reactant haptoglobin were observed again in both active and control groups at day 1 post-treatment compared to pre-treatment values (Fig. 2, *P*<0.049

in all but the 1st and 9th doses in MD_GT and *P*<0.026 at doses 1,2,7,9 in MD_CON). These increases were slight and had always resolved between each dose (*P*>0.104). While unexplained sporadic high values were observed in three of eight MD_CON animals at D1d15 these did not correspond to an acute change post-dosing and were not coincident with changes in any other parameter. Similarly two of eight MD_GT animals had elevated levels prior to Dose 1 delivery which persisted at D1d1 (Fig. 2) but are unlikely related to dosing.

Successive doses of pGM169/GL67A did not lead to consistent or cumulative changes in clinical chemistry parameters such as liver enzymes, urea, amylase and electrolytes (data not shown). Serum creatinine levels were generally stable over the course of the study (Fig. S4) although a trend towards greater delta creatinine (from pre-day1 at each dose) was observed in the MD_GT group (Linear change in successive delta values: *P*<0.01). Despite this, only ~2% of the measurements were ever above the reference range and the levels at day 15 (D9d15) or 4 weeks after the final dose were lower than the original baseline for the MD_GT group.

3.3 Bronchoalveolar lavage (BAL)

BAL was performed at day 1 and at day 15 after Dose 1, 5 and 9 (Fig. 3). In the MD_GT group total BAL cell numbers were elevated relative to pre-treatment values at day1 after all doses but only significantly at Dose 1 (D1d1: *P*<0.001) (Fig. 3b): these had resolved by the respective day 15 time points. The change from baseline to day 1 in the MD_GT group was not exacerbated at either Dose 5 or 9 when compared to the change after Dose 1 (*P*>0.051). The percentage of neutrophils in the BAL of the MD_GT group was increased at day 1 following Doses 1, 5 and 9 (all *P*<0.006) (Fig. 3d): again these increases had resolved by day 15 after each dose. The change from baseline to day1 at Dose 5 was significantly higher than that at Dose 1 (*P*<0.001) but there was no additional increase at Dose 9 (*P*=0.850). A significant increase in BAL macrophage numbers was also observed in the MD_GT group at day 1 after Dose 1 (D1d1)(Fig. 3f) compared to baseline (*P*<0.001) but macrophage numbers also returned to baseline by D1d15. At the other two time points the BAL macrophage numbers were either lower than or not different from baseline. The day 15 values after Dose 1, 5 and 9 in the MD_GT group were all lower than baseline (*P*<0.002) and post mortem examination (PME) values, 4 weeks after the final dose. were indistinguishable from baseline (*P*=0.617, Fig. 3f). BAL lymphocytes

also increased compared to baseline at D1d1 (MD_GT group only:*P*<0.001: data not shown) but day 15 values at Dose 1, 5 & 9 were all lower than baseline.

3.4 Lung Function

Gas transfer (DLCO) increased over the course of each delivery period (Fig. 4a) in both groups with no cumulative time-dependent change observed. An overall upward trend was noted in alveolar volume (VAeff) measurement in MD_CON and MD_GT animals over the course of the study (*P*<0.001, Fig. S5) and against this background there were occasional decreases over individual delivery periods at later doses in both MD_CON (D7, *P*=0.017) and MD_GT (D5, D7, *P*<0.028). However, no pGM169/GL67A-specific effect was observed. Lung Compliance (Cmlsr: Mean Least Squares Regression) also showed a statistically significant overall upward trend in both MD_CON and MD_GT (*P*<0.031, Fig. S6), but again, there was no pGM169/GL67A-specific effect observed in either pre- or post-dose data (*P*>0.175). In contrast, Functional Residual Capacity (FRC) and Lung Resistance (Rmlsr) measurements showed no overall time-dependent change (Figs. S7-S8).

Changes identified as a consequence of each period of anaesthesia and/or aerosol delivery (Pre-Post) were consistent between groups. Maximum Expired Flow (MEF) values did not differ between MD_CON and MD_GT (Fig S9). While a downward linear trend in successive pre-dose values was observed for both groups (*P*<0.014), there was no effect of the pGM169/GL67A at days 1 or 15, when measured after Dose 1, 5 and 9. Pre-dose values for Total Expired Volume (TotExpVol) showed a significant linear time-dependent increase over the course of the study in both groups (*P*<0.003) (Fig. S10). No pGM169/GL67A-related changes were observed in post-treatment measures at day 1 and 15 after Dose 1,5 or 9. No consistent changes were observed in either group for forced expiratory volume in 0.5 s (FEV 0.5) (Fig. S11) but incremental time-dependent decreases were apparent when expressed as a fraction of TotExpVol (FVC) (Fig. 4b) in both MD_CON and MD_GT groups. (linear change for pre-dose values *P*<0.001). Overall there were no pGM169/GL67A-related changes in expired flow or volume.

3.5 Multiple Breath Washout (MBW)

MBW is a sensitive test of predominantly smaller airways in man [13], and we therefore applied this technique for the first time to our sheep model. There was no difference between pre-Dose 9 and the 28-day recovery values for either the lung clearance index (LCI) (Fig. S12) or the functional residual capacity (FRC) data for the MD_GT or MD_CON (P>0.518) and neither group was significantly different to the data from the sentinel group (P>0.597) indicating that there was no adverse effect of the test item on LCI measurements.

3.6 Histology and lung morphometry

Blinded histopathological analysis revealed no difference between MD_GT, MD_CON and Sentinel animals. Some very minor changes were noted in single sheep including rare and mild focal fibrosis (Figs. 5a,c) in a MD_GT animal and mild cellular infiltrates with minimal interstitial fibrosis (Figs. 5b,d) in a MD_CON animal, although these were considered to be incidental. Overall there was nothing to suggest a harmful consequence of the repeated delivery of pGM169/GL67A. Serial sections from the same blocks as the H & E sections above were stained with Masson's trichrome. Blinded analysis confirmed the presence of mild collagen deposition in the previously described areas of fibrosis but did not indicate any widespread interstitial fibrosis (Fig. S13). There was no evidence of a consistent effect of treatment with pGM169/GL67A. The Oil Red O stain on frozen sections showed distinct red staining restricted to peribronchial adipocytes (fat cells) (Fig. S14). The number of fat cells are within normal limits and not unexpected in this location. Occasional free red lipid droplets were noted in animals from both groups but no red foamy macrophages. Rarely, red droplets were observed in some capillaries or in alveolar walls, suggestive of absorption of lipid into the bloodstream but this observation was not restricted to the MD_GT group. Overall the Masson's trichrome and Oil Red O stains provided no evidence of a pGM169/GL67A-related effect.

Blinded morphometric analysis of slides from all three groups (Fig. 6) found no significant differences between the groups in alveolar septal wall thickness (Fig. 6a) or in area fraction (% Area : Fig. 6c), a measure of the ratio between lung tissue and airspace. A difference was observed in the mean linear intercept (Lm) values (Fig. 6b), an indicator of alveolar size, however it was the MD_CON group that had a higher Lm value than both Sentinel and MD_GT (P<0.031) groups thus no pGM169/GL67A specific effect was observed on lung morphometry. On blinded analysis of H & E

stained sections from heart, kidney, liver and spleen there were no persistent histological lesions 4 weeks after final delivery that could be attributed to effects of treatment with nine doses of pGM169/GL67A (data not shown).

3.7 Molecular Analyses

Bronchial brushings (BBr) and tissue (Tx) samples were analysed by quantitative PCR for pGM169 and endogenous ovine *CFTR* (oCFTR) DNA as well as for pGM169 mRNA and oCFTR mRNA. Plasmid pGM169 DNA was detected in all BBr samples from the MD_CON and MD_GT animals (Fig. 7). However the day 1 post-dose levels of pGM169 DNA in the MD_GT samples were two to three orders of magnitude higher than the levels of pGM169 DNA detected in either the MD_CON samples (P<0.001) or the preliminary samples from the MD_GT animals (P<0.001). In the MD_GT group at necropsy, 53/64 BBr (median value 87% pGM169/oCFTR DNA, $1.58x10^2$ copies/ng DNA) and 77/96 Tx samples (median value "positive but not quantifiable" either expressed as % pGM169/oCFTR DNA or copies/ng DNA) were positive for pGM169 DNA. Ovine *CFTR* mRNA was detected in 285/288 of the MD_CON group and 288/288 of the MD_GT group samples.

No pGM169 mRNA was detected in any sheep at any time point in the MD_CON group. Plasmid-derived gene expression was detected in 12% of BBr samples at day 1 after the first dose, 34% of BBr at day 1 after the 5th dose and 12% of BBr at day 1 after the ninth dose. By day 28 after Dose 9, only 2/96 lung tissue (Tx) samples had detectable pGM169mRNA and no pGM169 mRNA was detected in the BBr. Table 1 indicates the MD_GT samples in which pGM169 mRNA was detected 1day after each dose. It is interesting to note that positive BBr samples were obtained from two sheep (MD1,MD 16) at Day 1 after doses 1,5, and 9 (12 of the the 19 positive BBr came from these two sheep), from four sheep at one or two of the day 1 timepoints and there were two sheep (MD2, MD18) that were always negative at the day 1 timepoints.

4. Discussion

In this study we report for the first time the outcome of nine monthly aerosol administrations of a non-viral gene therapy formulation, to a large animal model with lungs of a similar size and structure to

those of humans. We have previously used this model system to compare the efficacy of three gene transfer agents in studies [3] that informed the selection of the current candidate formulation for a Multi-dose clinical trial in CF patients. The primary goal of these studies was to determine whether the mild acute adverse responses observed in our previous studies would lead to cumulative or chronic changes in the lung after multiple administrations. In this study, the average dose in the nebuliser was 0.48mg pGM169/kg and 2.68mg GL67A/kg. This represents an approximately two-fold greater dose than is being used in the Multi-dose Trial (based on a 60 kg patient) and approximately 2-fold lower than the maximum dose used in previous studies in sheep and humans, with the intent to minimise or abolish the mild and transient acute response noted in those studies.

Repeated gene delivery by aerosol was well tolerated by the sheep with no obvious clinical signs throughout the study. The majority of changes observed were mild and were observed in both anaesthetic controls (MD_CON) and treated (MD_GT) animals. For example a small decrease in animal bodyweight and increases in haematological parameters such as RBCs, WBCs and haemoglobin were observed at day 1 and are likely a consequence of the anaesthetic procedure. The overall downward trend in blood lymphocytes and WBC numbers observed in MD_CON and MD_GT groups was also seen in the sentinel animals, suggesting that these are normal changes in maturing animals. Plasma concentrations of the serum acute phase protein haptoglobin are elevated as part of the host innate immunity in response to inflammatory cytokines and therefore are indicative of inflammation. The increases in haptoglobin observed at day 1 after delivery were also observed in the MD_CON group and were less than 2-fold suggesting a mild response to the anaesthetic procedure rather than to the gene transfer agent as changes of more than 50-fold have been reported in response to lung damage in sheep [14]. All of these changes resolved and were not exaggerated by repeated dosing.

The absence of a gene transfer-specific increase in haptoglobin in the current study using a 10 ml aerosolised dose contrasts with our previous results demonstrating a significant gene transfer agent effect with a 20ml aerosolised dose of pDNA/GL67A complex [3] and suggests that the lower dose has an improved safety profile. Although a significant upward trend was observed in serum creatinine levels from pre-treatment to day 1 in the MD_GT group the absolute increase in values was relatively small, the majority of the values (~98%) being within the normal reference range for sheep (40-150µmol/l) and the final values after the 4 week recovery period were lower than the baseline levels. It

is important to note that none of the mild post–dose changes increased in severity with successive doses.

The main area in which gene transfer-specific effects were observed was in relation to changes in BAL cellularity measured after Doses 1, 5 and 9. BAL was only performed at these time points to limit the number of anaesthetic periods during the study. Changes in cellularity manifested as increases in total BAL cell number, the percentage of neutrophils, total macrophages and total lymphocytes. Importantly, responses were not exaggerated by repeated delivery and the observed changes had always fully resolved by day 15 after a delivery. The magnitude of the changes in BAL cells after nine doses was not more severe than those previously observed in the sheep lung at day 1 after a single 20ml (~1 mg /kg / dose pDNA and ~5.2mg GL67A / kg / dose) dose [3]. Interestingly, although the percentages of macrophages and lymphocytes in the BAL always showed a decrease due to the large increase in neutrophils, an increase in absolute BAL macrophage and lymphocyte numbers was observed but only after Dose 1. Unlike previous studies in the mouse [2], delivery of CpG-free plasmid/lipid complexes to the sheep lung did not completely abolish the transient inflammatory cell response in BAL samples. Similarly, the use of CpG-free plasmid DNA did not completely eliminate the mild and transient “flu-like” symptoms observed in patients [4] and suggests that sheep may be more similar to humans in terms of responses to pDNA/lipid delivery, than mice.

Lung function parameters (DLCO, VAeff, FRC, Compliance, Resistance) measured immediately pre- and post-delivery showed no changes attributable to delivery of pGM169/GL67A. Mild changes were observed either as a response to the anaesthetic/ventilation period or perhaps as a consequence of normal development/maturation of the lungs over the course of the study. For example, gas transfer (DLCO) might have been expected to decrease if lung function was compromised by delivery; DLCO actually increased during each delivery period in both groups. The mechanism for this increase is unknown but we speculate that an increase in pulmonary capillary blood volume associated with whole body negative pressure ventilation may contribute to this phenomenon. Forced expiration analysis also demonstrated that there were no pGM169/GL67A treatment-related effects on the animals. Changes such as the steady increase in total expired volume (TotExpVol or FVC) over time, the consequent “decline” in FEV 0.5 when expressed as a proportion of TotExpVol (FVC) or the overall decline in MEF, were observed in both groups.

These findings may also be a consequence of normal development/maturation in the lung since the final values after the 4-week recovery period were not different to those of the sentinel group. Animal welfare considerations precluded a more acute evaluation of spirometry that would align with data available from clinical trials. Such clinical data indicate a drop in FEV_1 in patients over the 6 hours following delivery that resolves by day 1 [4]. Lung Clearance Index is known to be elevated in CF patients with normal spirometry and therefore is believed to be a more sensitive predictor of deteriorating lung function, particularly in the smaller airways [13]. Multiple Breath Washout analysis to derive LCI values was performed on animals immediately prior to delivering Dose 9 and again after the 4-week recovery period at the end of the study. There were no significant differences in the LCI or FRC data obtained from this protocol between the MD_CON, MD_GT and sentinel groups.

The overall conclusion from histological analysis at 4 weeks after the final dose indicated that there was nothing to suggest a harmful consequence of the repeat deliveries and the associated acute neutrophilic response noted in the BAL. In the lungs, minimal/mild lymphoplasmacytic (and sometimes eosinophilic) inflammation was observed around bronchioles and some blood vessels. Mild to moderate bronchus-associated lymphoid tissue (BALT) hyperplasia was also observed but these features were not confined to MD_GT animals and are consistent with background immunosurveillance and/or indicative of previous parasite exposure. Alveolar histiocytosis was also common to all three groups (four in the Sentinel group, five in the MD_CON group and six in the MD_GT group). These macrophages were very occasionally mildly vacuolated but were not distinctly foamy. Mild alveolar histiocytosis is not unusual in the ovine lung, especially, as in this case, when the sheep are not specific pathogen free (SPF) animals, or maintained in climate- or pathogen-controlled housing.

There were no notable features observed on slides stained with Oil Red O to suggest an accumulation of lipid related to treatment. In contrast in a parallel murine study the high-dose (13.1 mg pGM169 / kg / dose and 74 mg GL67A / kg / dose) and medium-dose (4.6 mg pGM169 / kg / dose and 26 mg GL67A / kg / dose) treated animals showed red foamy macrophages persisting after 12 doses of pGM169/GL67A (EWFW Alton *et al*, manuscript submitted) These doses in mice represent an approximate overage of 60- and 20-fold relative to the anticipated human dose (based on a 60 kg patient) for the high and medium dose cohorts, respectively. In keeping with the current study red-stained foamy macrophages were not observed in mice treated with the lowest dose 1.2mg pGM169 /

kg / dose and 6.7 mg GL67A / kg / dose which is still over two-fold higher than the dose administered to the sheep (0.5mg pGM169 / kg / dose and 2.6mg GL67A / kg / dose).

Other changes noted included occasional and mild localised pleural fibrosis (to the same extent in all groups), likely due to resolving pleuritis. Similarly, there was no adverse effect of repeated gene delivery observed on lung morphometry in terms of changes in alveolar septal thickness or alveolar size to indicate remodelling. In the sections from heart, kidney, liver and spleen, there was nothing to indicate an adverse effect of treatment. Lesions present in all groups were likely incidental and consistent with background inflammation or immunosurveillance.

We have previously shown that this method of delivery results in widespread deposition of plasmid in the lung. Despite the presence of environmental pGM169 leading to contamination of pre-treatment and MD_CON samples during sample harvesting and/or processing, pGM169 DNA was clearly detectable well above background in all post-treatment BBr samples at day 1 after each dose, and in the majority of samples at day 28 after the final dose. There was no evidence of pGM169 DNA accumulation in the lung, with increasing doses of pGM169/GL67A. Plasmid-derived hCFTR mRNA was detected in BBr after all doses but expression levels even after repeated administration remained comparatively low and did not appear to persist when analysed 28 days after Dose 9. These results contrast with data obtained in mice following delivery of pDNA with the luciferase reporter gene driven by the same promoter where a persistent level of gene expression was observed beyond the 28-day timepoint [2] and in humans where prolonged correction of the chloride transport defect was observed [4]. These differences could be due to species-specific effects in the performance of the hCEFI promoter/enhancer, in efficiency of gene delivery, or in the type or location of cell samples harvested for analysis. Such differences could also be due to the difficulty in detecting vector-specific transcripts following gene transfer. The proportion of samples testing positive for plasmid-derived mRNA in other published clinical trials that have delivered plasmid DNA with GL67A have reported similar difficulties [6, 15], even when there is evidence of functional correction [5]. Positive mRNA samples were consistently generated from two of the eight individual sheep at day 1 following Dose 1, 5 and 9 and four animals were positive at some but not all of the day 1 timepoints. The remaining two sheep gave no positive results ; this may suggest, as appears to be the case for humans, that perhaps unsurprisingly in an outbred population, there are individuals that are more susceptible to gene transfer than others.

5. Conclusions

Inhalation exposure of sheep to nine doses of pGM169/GL67A aerosol was well tolerated with no clinical symptoms. The gene expression data suggests inherent differences in susceptibility between individual animals and understanding this 'stratified' response, if reproduced in the Multidose Trial in CF patients, may be crucial in the progression of clinically relevant non-viral gene transfer. The safety data indicate that the only test item-related local effect in the lung was the increase in BAL neutrophils. This response has been described previously and remains despite the use of CpG-free plasmid DNA. Importantly, the BAL neutrophil response observed after 9 doses is no more severe than that observed after a single dose and had resolved within 15 days. Any observed changes in lung function or spirometry were also observed in control animals therefore likely reflect an effect of the prolonged anaesthetic period or normal lung maturation in developing animals. Lung clearance index values in treated animals were no different to controls. Histological analysis revealed no changes related to repeated administration and no accumulation of lipid in macrophages. Parameters such as bodyweight, haematology or clinical chemistry gave no indication of a systemic response to repeat administration of pGM169/GL67A, Overall there were no adverse responses observed that would undermine confidence in progressing towards a similar Multidose study in CF patients.

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Captions

Fig. 1. Diagram illustrating study outline. Where practical, measurements and observations were performed immediately before (Pre-) and immediately after (Post-) each delivery period. Sampling procedures not requiring anaesthetic were performed at day 1 following every delivery. Arrows indicate deliveries or invasive sampling days involving anaesthetic. Broken arrow: Baseline, Black arrows: pGM169/GL67A delivery (MD_GT Group) or anaesthetic only (MD_CON group). Grey Arrows: d1 Sampling (Invasive). Arrowheads: d15 Sampling. White Arrow: Necropsy.

Fig.2. Plot showing longitudinal serum haptoglobin levels in: anaesthetic control (MD_CON), pGM169/GL67A (MD_GT) and Sentinel (SEN; D1d1, D5d1, D9d1 & PME only) animals. Symbols represent group mean +/- 95% CI. Baseline : 4 weeks before dose 1. Pre : immediately before delivery. d1 : one day after delivery. d15 : 15 days after delivery. PME : end of 4-week recovery period from dose 9.

Fig. 3. Scatterplots showing bronchoalveolar lavage cell data in anaesthetic control (MD_CON) (a, c, e) and pGM169/GL67A (MD_GT) (b, d, f) animals. Figures show data for total BAL cells (a,b), % Neutrophils (c,d) and macrophage numbers (e,f). Symbols represent individual animal values: bar represents group mean. Pre1: 4 weeks before dose 1. d1: one day after delivery. d15: 15 days after delivery. PME: end of 4-week recovery period from dose 9.

Fig. 4. Plot showing longitudinal lung function data for anaesthetic control (MD_CON), pGM169/GL67A (MD_GT) and Sentinel (SEN; PME only) animals. (**a**) Gas Transfer (DLCO (SB)) factor (**b**) Forced Expiratory Volume (FEV0.5) as proportion of FVC.Symbols represent group mean +/- 95% CI. Pre: immediately before delivery. Post: immediately after delivery. d1: 1 day after delivery. d15: 15 days after delivery. PME: end of 4-week recovery period from dose 9.

Fig. 5. Haematoxylin and eosin-stained sections from lungs illustrating some of the minor and focal changes observed in single animals from the pGM169/GL67A-treated (MD_GT; **a,c**) and anaesthetic control (MD_CON; **b,d**) sheep 4 weeks after the final dose. Panel (**c**) is ahigher magnification of the rare foci of fibrosis (arrow) from inset in panel (**a**). Panel (**d**) is a higher magnification of the mild cellular infiltrate containing macrophages and neutrophils (arrows) from inset in panel (**b**). Scale bars. (**a,b**) 200µm, (**c,d)** 100 µm,

Fig. 6. Scatterplots showing lung morphometry data from Masson's Trichrome stained sections of lung from anaesthetic control (MD_CON), pGM169/GL67A (MD_GT) and Sentinel animals. Individual plots show septal

wall thickness (**a**) mean linear iIntercept (**b**) and area fraction (**c**). Symbols represent individual animal values. Bar represents group mean.

Fig. 7. Plot showing levels of pGM169 DNA as a percentage of endogenous oCFTR DNA in live brushing samples (**Live BBr**) or brushings (**BBr**) and tissue (**Tx**) samples harvested at post-mortem. MD_CON: anaesthetic control, MD_GT: pGM169/GL67A. Symbols represent individual bronchial brushing (BBr) or Tissue (Tx) sample values. Black bars represents group median. Pre = 4 weeks before dose 1. d1: 1 day after delivery. d28: 28 days after delivery of dose 9. PBNQ = positive but not quantifiable. ND: not determined.

Table 1. Summary of samples positive for pGM169 mRNA in individual animals at each of the time-points sampled in the pGM169/GL67A-treated animals (MD_GT)

	Brushings				Tissue
Sheep ID	**D1d1 (32)**	**D5d1 (32)**	**D9d1 (32)**	**D9d28 (64)**	**D9d28 (96)**
MD1	2	2	2	0	0
MD2	0	0	0	0	1
MD3	1	2	0	0	0
MD4	0	1	0	0	0
MD16	1	4	1	0	0
MD18	0	0	0	0	0
MD19	0	1	0	0	0
MD24	0	1	1	0	1
TOTAL	**4**	**11**	**4**	**0**	**2**
Median LLQ	**0.63%**	**0.88%**	**0.24%**	**0.13%**	**0.11%**

No samples were positive for pGM169 mRNA in the pre-treatment samples or at any time-point in the anaesthetic control group (MD_CON). D1d1 = dose 1 day 1, D5d1 = dose 5 day 1, D9d1 = dose 9 day 1, D9d28 Dose 9 day 28. MD denotes animal identification number for the study. Figure in brackets represents the total number of samples analysed.
LLQ: lower limit of quantification (pGM169 mRNA as a percentage of endogenous CFTR mRNA)

Figure 01

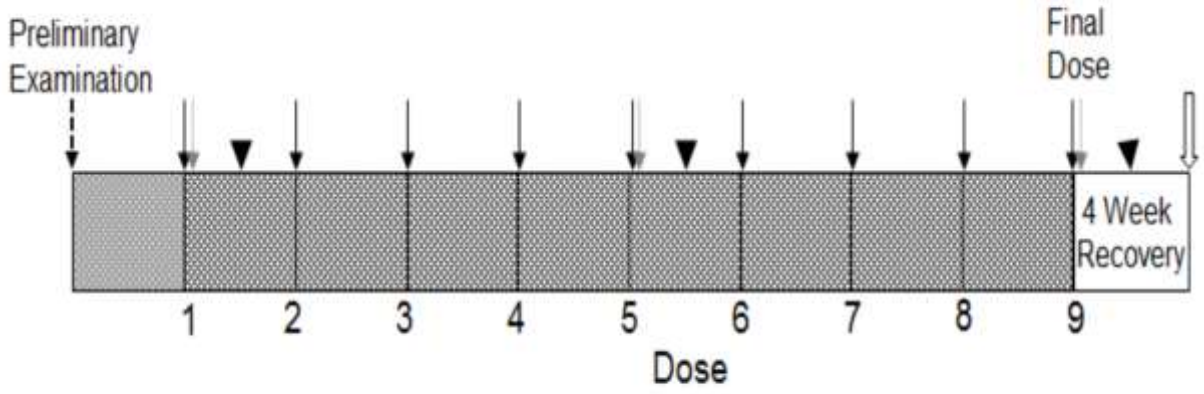

Preliminary
Examination
Final
Dose
4 Week
Recovery
1
2
3
4
5
6
7
8
9
Dose

Figure 02

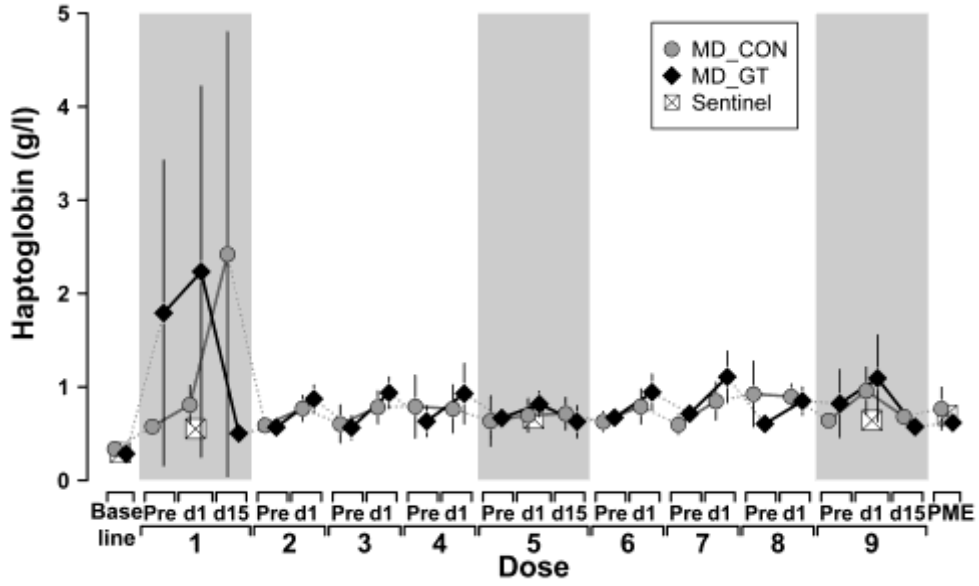

Haptoglobin (g/l)
0
1
2
3
4
5
MD_CON
MD_GT
Sentinel
Base line
Pre d1 d15
Pre d1
Pre d1
Pre d1
Pre d1 d15
Pre d1
Pre d1
Pre d1
Pre d1 d15
PME
1
2
3
4
5
6
7
8
9
Dose

Figure 03

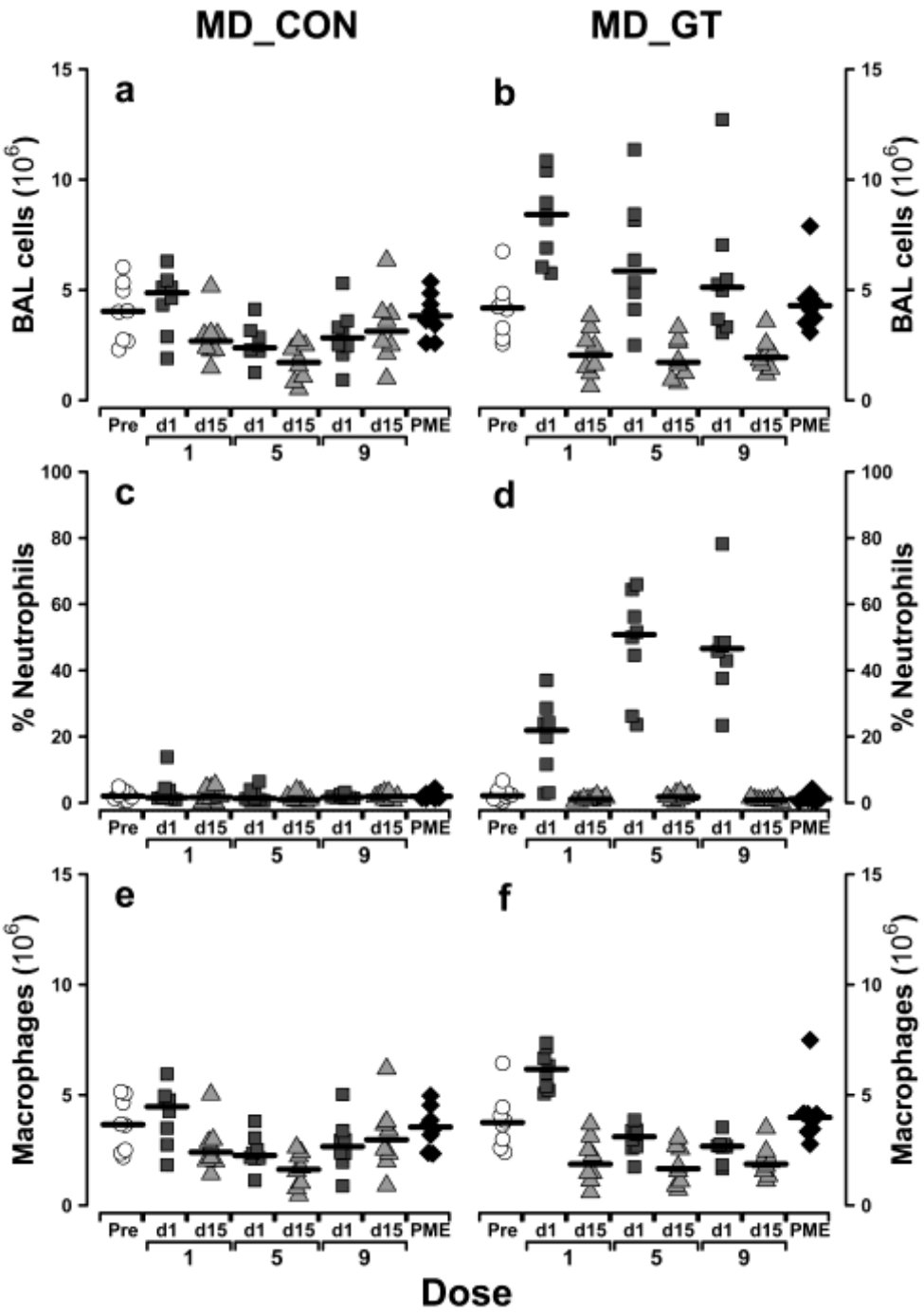

MD_CON
MD_GT
a
b
c
d
e
f
BAL cells (10^6)
% Neutrophils
Macrophages (10^6)
Pre
d1
d15
PME
1
5
9
Dose

Figure 04

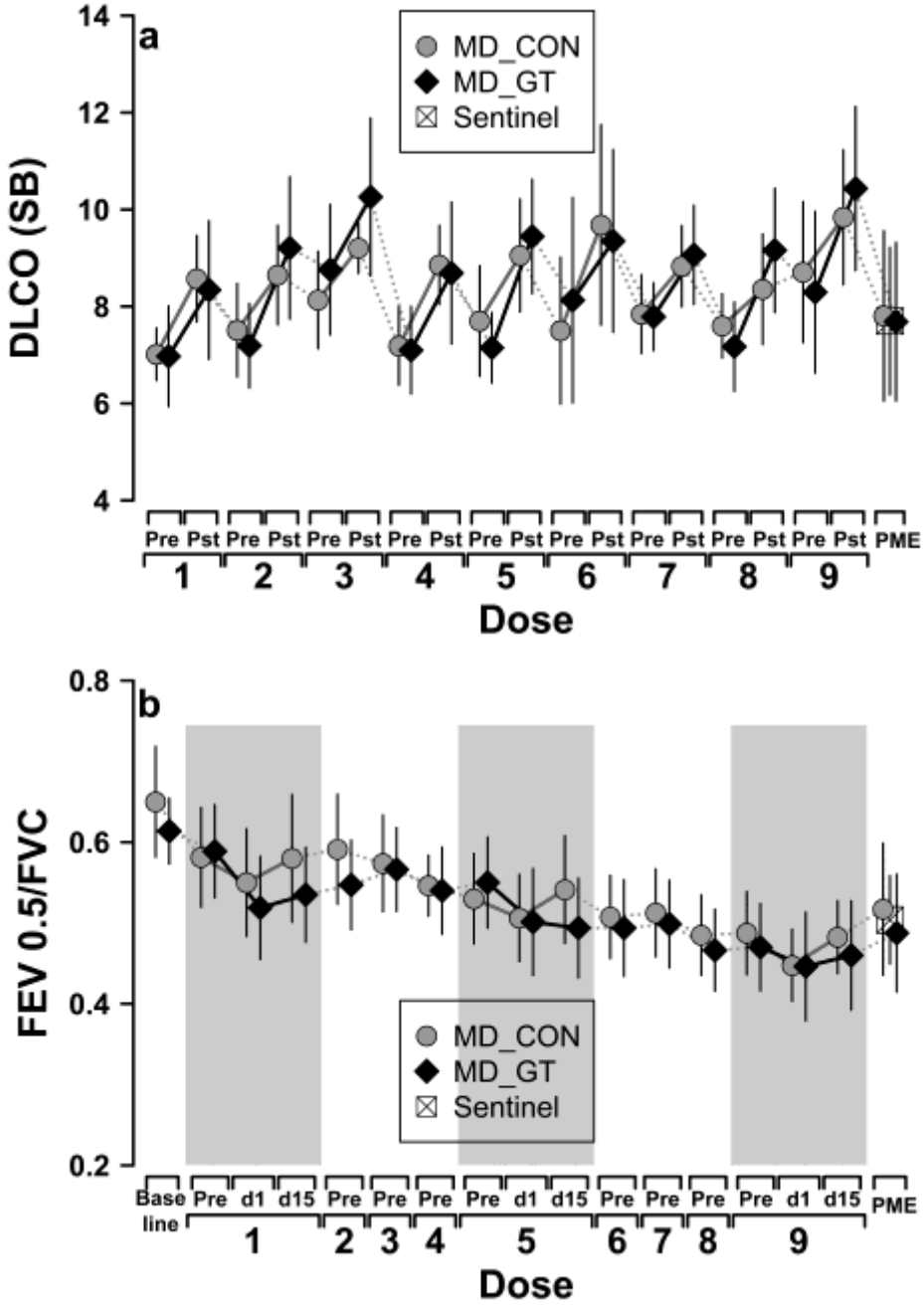

a
DLCO (SB)
14
12
10
8
6
4
MD_CON
MD_GT
Sentinel
Pre Pst Pre Pst Pre Pst Pre Pst Pre Pst Pre Pst Pre Pst Pre Pst Pre Pst PME
1 2 3 4 5 6 7 8 9
Dose
b
FEV 0.5/FVC
0.8
0.6
0.4
0.2
MD_CON
MD_GT
Sentinel
Base line
Pre d1 d15 Pre Pre Pre Pre d1 d15 Pre Pre Pre Pre d1 d15 PME
1 2 3 4 5 6 7 8 9
Dose

Figure 05

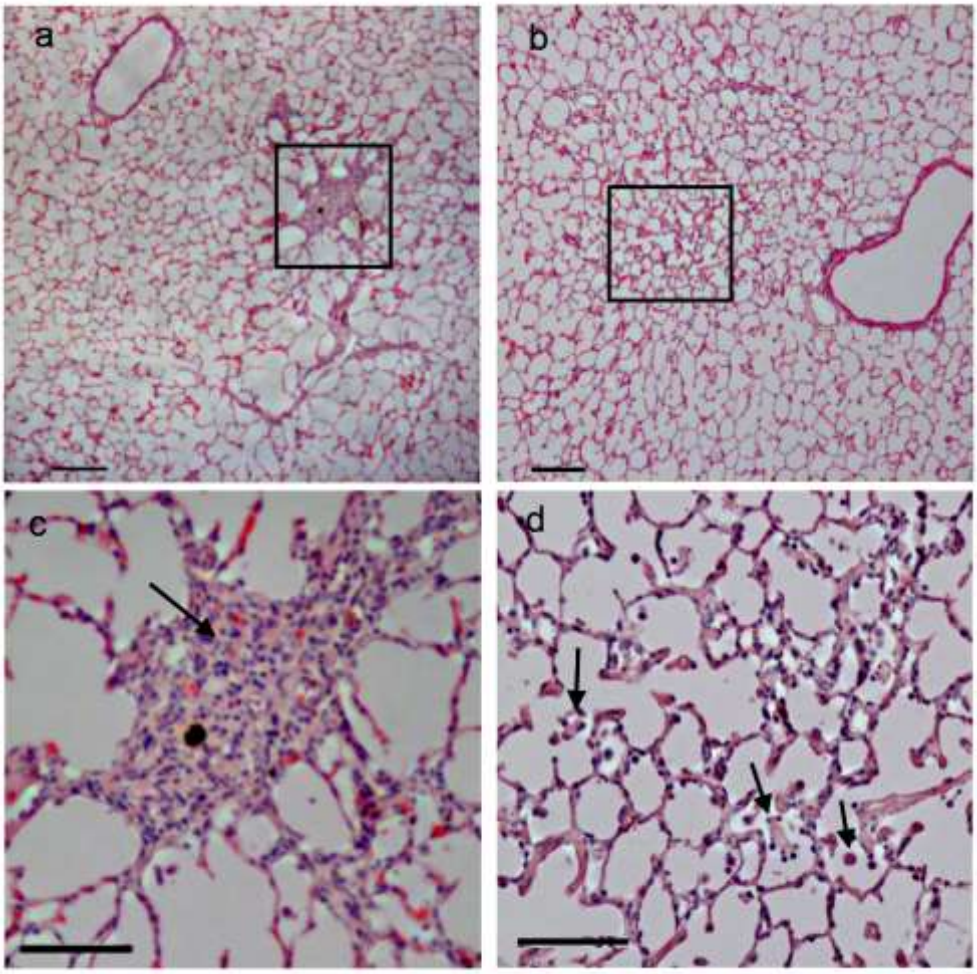


Figure 06

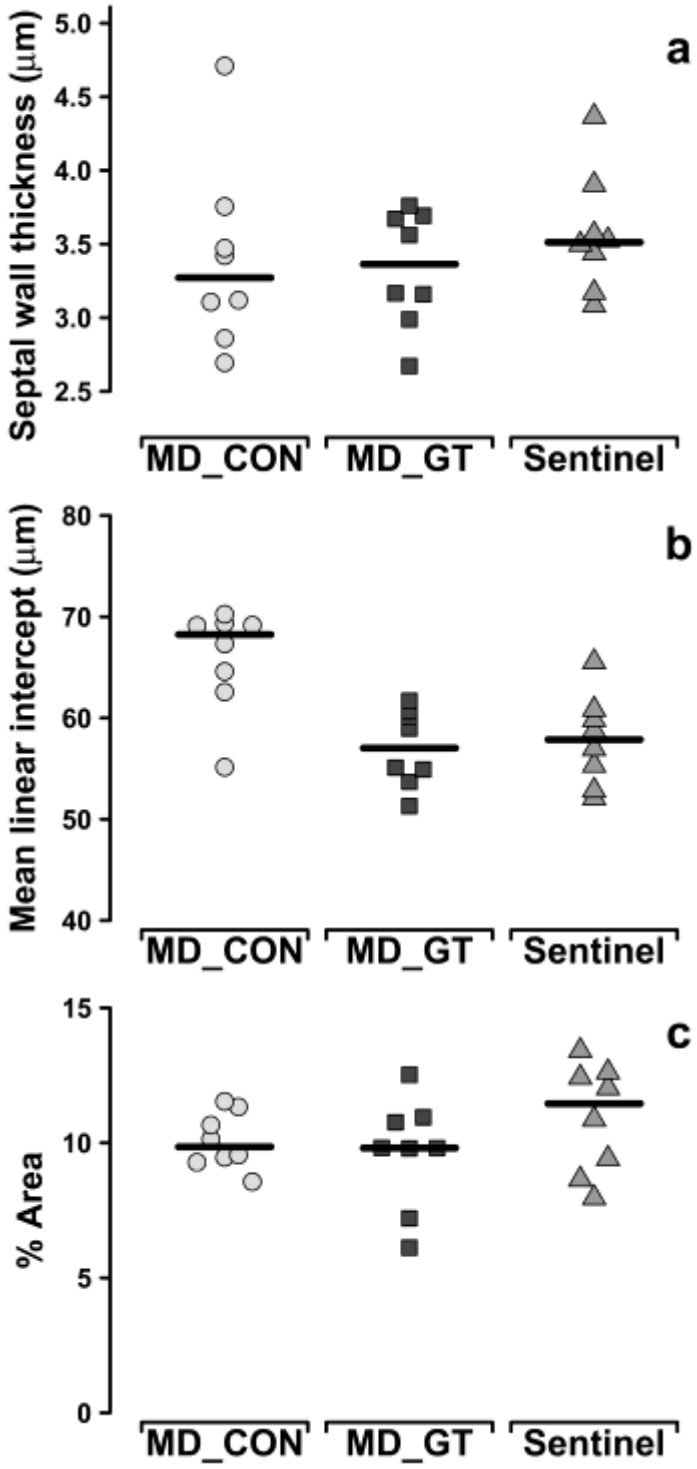
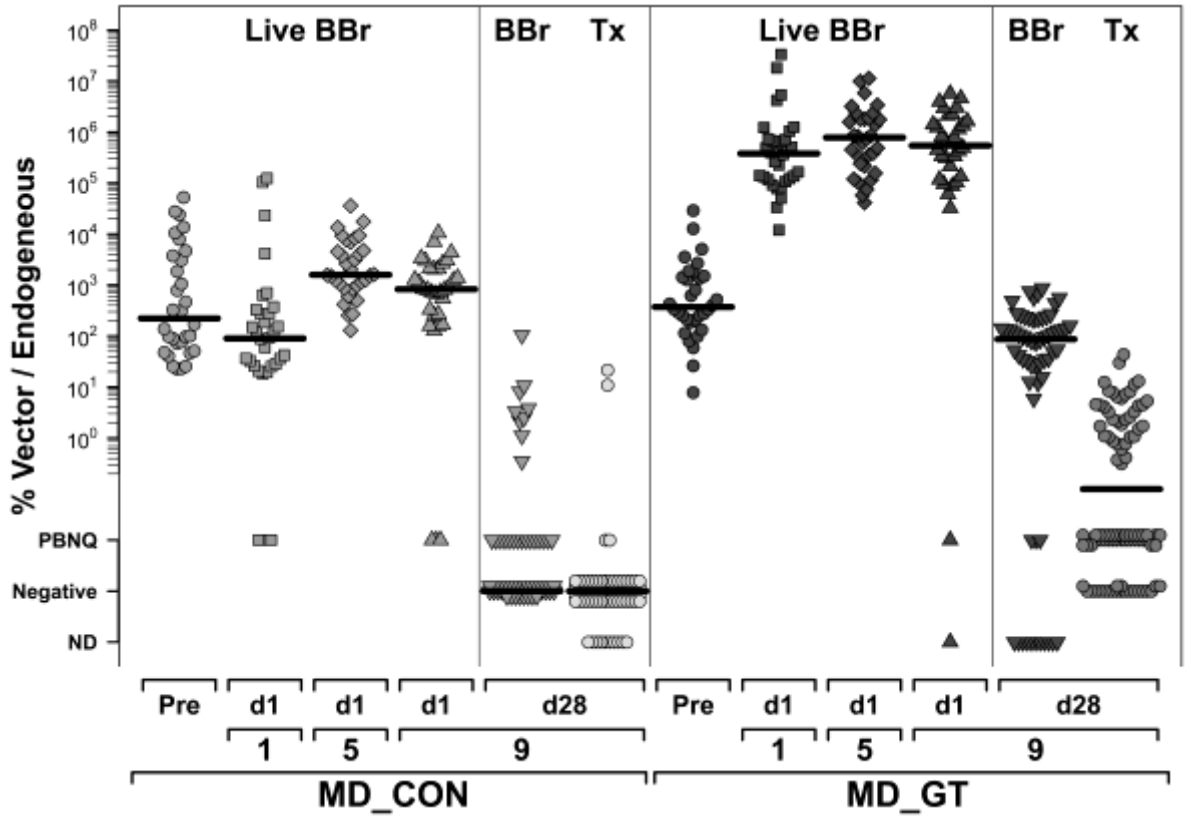

a
Septal wall thickness (μm)
5.0
4.5
4.0
3.5
3.0
2.5
MD_CON
MD_GT
Sentinel
b
Mean linear intercept (μm)
80
70
60
50
40
MD_CON
MD_GT
Sentinel
c
% Area
15
10
5
0
MD_CON
MD_GT
Sentinel

Figure 07


Live BBr
BBr
Tx
Live BBr
BBr
Tx
% Vector / Endogeneous
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
10^0
PBNQ
Negative
ND
Pre
d1
d1
d1
d28
Pre
d1
d1
d1
d28
1
5
9
1
5
9
MD_CON
MD_GT